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The biologic response of human anterior cruciate ligamentocytes on collagen-patches to platelet-rich plasma formulations with and without leucocytes

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Abstract

Due to the poor self-healing capacities of the anterior cruciate ligament, previous primary repair attempts have failed. To enhance biologic healing, platelet rich plasma and collagen scaffold have shown promise in animal models. Platelet rich plasma (PRP) is already used in several clinical applications although outcomes are quite debated. The purpose of this study was to examine the effects of different PRP formulations during 21 days: with leucocytes and pure PRP on human anterior cruciate ligament-derived ligamentocytes grown on collagen patches in 3D cell cultures *in vitro*. Three experimental groups were formed: 2.5% leucocyte rich PRP, 2.5% pure PRP, 20% leucocyte rich PRP, a negative control, and a positive control. Cell proliferation, cell phenotype on mRNA transcript level, and extracellular matrix production (total collagen and glycosaminoglycan content) were evaluated. DNA content and metabolic cell activity increased significantly in all groups on day 21 compared to day 7, except in the negative control. No changes in extracellular matrix production were detected. Different catabolic genes were induced depending on the concentration of leucocyte rich PRP. PRP with and without leucocytes treated anterior cruciate ligamentocytes significantly increased cell proliferation but not extracellular matrix production. However, the specific activation of different catabolic genes was dependent on the relative content of leucocytes.

Keywords: anterior cruciate ligament; platelet-rich plasma; leucocyte; proliferation

INTRODUCTION

Anterior cruciate ligament (ACL) lesions are one of the most mundane sport injuries, estimated to impact 100,000 to 200,000 people yearly in the U.S.[1] Due to the poor healing capacity of the ACL, injuries have to be treated frequently with graft reconstruction to restore gross knee stability. Because of the drawbacks of this procedure such as donor side morbidity, deteriorated proprioception and a controversial preventative effect on osteoarthritis development[2] new treatment options are sought, which enhance the healing response of the native ACL.[1]

Platelet rich plasma (PRP) contains highly concentrated thrombocytes, which are known to promote wound healing upon injury.[3] Biocompatibility, an autologous character and the secretion of a tremendous number of growth factors render PRP as the perfect prerequisite for biological enhancement of regeneration. It has gained popularity in the clinical treatment of sports related injuries because of its easy use and cost-effectiveness[4,5] although the function of PRP is very controversial depending on varying concentrations of blood components and targeted cell type.[2]

In vivo experiments on large animal models have demonstrated the anabolic effect on ligamentocytes (LC) treated with 3D-collagen-PRP scaffolds.[6-8] Based on the positive outcome of these studies the first human trial commenced recently[3] although exact cellular mechanism are still elusive. One key consideration when using PRP is the release of bioactive factors dependent on platelet concentration. Interestingly, high PRP concentrations result in reduction of LC proliferation, a suboptimal effect on function[9] as well as increased cell death.[4] In several studies the lowest examined concentration yielded the best results, which had a similar count to the baseline of whole blood or contained only platelet poor plasma.[4, 5] Furthermore, results are amplified, when ACL cells are treated with a combination of PRP and mononuclear cells, a type

of leucocytes.[12]

Our aim was to fully elucidate whether higher dilution of PRP could influence human ACL cells and if the addition of leucocytes would differ results. The hypothesis was that in the presence of even low concentrations of PRP human LCs would alter cell proliferation, extracellular matrix (ECM) production and expression of anabolic genes. Additionally, leucocytes added to PRP would increase the anabolic effect.

MATERIAL AND METHODS

ACL-LC isolation

Fresh human ACL tissue was obtained from five young male donors (age range 26-32 years) with written consent undergoing ACL reconstruction surgery with approval of the ethical committee (#201/2015) of the Canton of Berne (dated 07/21/2015). On the same day tissue was cut into ~three mm^3 pieces, washed with 1 x phosphate buffered saline (PBS) and digested overnight in 230 U/mg collagenase 2 (Worthington, UK) in an incubated humidified environment (37°C, 5% CO_2, 95% humidity), under constant shaking at 10 RPM. On the next day, the released cells were filtered with a 100 µm cell strainer (BD Falcon, Switzerland) and seeded at a density of 1,000 cells/cm^2 for monolayer expansion in high glucose Dulbecco's Modified Eagle's Medium (HG-DMEM, Gibco, Switzerland) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, Switzerland) and 1% penicillin/streptomycin (P/S, Sigma-Aldrich) as previously described.[6]

PRP Production

A total of 60 ml fresh blood samples per PRP group were collected from young human male donors (27 ± 4.1 years, mean age ± SD) and clotting was artificially prohibited through acid citrate dextrose (Biomet Biologics, Inc., Dietikon, Switzerland). The ethic committee of the University of Berne as well as the donors gave their consent. The local authorities ethically approved this protocol. Subsequently, the blood samples were centrifuged separating the different blood cell types based on weight according to two different protocols: i) instructions of the Platelets Matter/GPS® III commercial Kit (Biomet Biologics) and ii) according to Yoshida *et al.*[5] The GPS kit yielded a PRP rich in leucocytes (L-PRP), whereas the protocol of Yoshida *et al.*[5] contained purely platelets (P-PRP). PRP was activated with 1.5 U/ml Thrombin (from Tisseel Kit, Baxter inc., Vienna, Austria) before application. Cell composition of the PRP was determined using an automatic hematology analyzer, the ADIVA® 2120i System (Siemens, Zurich, Switzerland)

LCs proliferation trial

Our trial was designed to find out if a higher dilution of PRP could also affect LCs. LCs were seeded in a 6-well plate at a density of 1 x 10^3 and cultured in HG-DMEM containing L-PRP at various concentrations: 0%, 2.5% (0.1 x), 5% (0.2 x), 20% (0.8 x), 40% (1.6 x). A five-fold increase on day 14 ($p = 0.04$) and six-fold on day 21 ($p < 0.0001$) was noted when 20% PRP was compared to the negative control. In the 2.5% L-PRP group a five-fold difference was noted on day 21 compared to HG-DMEM ($p = 0.0002$). 5% L-PRP was significantly augmented on day 7 compared to HG-DMEM ($p = 0.0039$) but decreased afterwards. Consequently the 2.5% and 20% L-PRP groups, which performed the best on day 21, were selected for further analysis.

3D Cell cultures

The 12-well plates (Falcon, Becton Dickinson, inc. Allschwil, Switzerland) were coated with 2% agarose (Sigma-Aldrich, Buchs, Switzerland) inhibiting cells attaching at the bottom of the well during 3D culture. FDA-approved collagen type I/III patches (ChondroGide™, Geistlich, Wohlhusen) were cut with a six mm biopsy punch (Kaimedical, Polymed Inc., Glattburg, Switzerland) and placed onto the agarose. 3 x 10^4 LCs (N = 5 donors) at passage two were seeded on the rough side of collagen patches.[6] All experimental groups were run in biological replicates n = 2 per donor. The cells were incubated for 24h in HG-DMEM + 10% FCS to allow cell adherence to the patch. PRP was separated from the well by co-culture inserts with a high-density PET membrane as seen in Fig. 1 (0.4 µm pore size, Falcon). During the 3D cell culture PRP was freshly produced from fresh human blood samples and PRP and culture medium was refreshed every 2-3 days. Outcome measurements were performed at day 7, 14 and 21.

Cell Viability Assay

Resazurin Sodium Salt (Sigma-Aldrich) was added to each well of the 12-well plate (Falcon. BD, Allschwil, Switzerland) and wells were incubated at 37°C, 5% CO_2 for three hours on a shaker at 10 RPM as previously described.[6] When resazurin enters the cell it will be reduced to its red fluorescent form resorufin by mitochondrial enzymes. The fluorescence is proportional to the number of mitochondrial active cells.[7] The relative fluorescent units (RFUs) were then quantified with Softmax M5 Pro Multi wavelength fluorescence reader (Molecular Devices, distributed by Bucher Biotec, Switzerland) at an excitation at 547nm and an emission wavelength at 582nm.

Quantification of DNA, glycosaminoglycan (GAG) and hydroxy-proline (HYP) content

Samples were dried for three days at 60°C, weighted and digested with papain (Sigma-Aldrich) for 16 hours. Subsequently DNA, GAG and HYP content were quantified. Quant-iT™ PicoGreen® (Invitrogen, US) was used to determine the DNA content according to the manufacturer's protocol. Calf thymus DNA (Sigma-Aldrich) served as standard. 1,9-Dimethyl-methylene-blue chloride dye (DMMB, Sigma-Aldrich) binds to GAG and precipitates and consequently absorbance can be measured to ascertain the GAG content. Bovine chondroitin sulfate (Sigma-Aldrich, Switzerland) served as a standard.[6] Acid hydrolysis and reaction with 4-dimethylamino-benzaldehyde (Sigma-Aldrich) and chloramine-T (Sigma-Aldrich) was used to determine HYP content which was read at an absorbance at 560nm. L-4-HYP (Sigma-Aldrich) was employed as standard.

RNA extraction and Quantitative Real-time polymerase chain reaction (qPCR)

A combined phase-separation and silicone membrane purification was used according to Reno *et al.*[8] Samples were snap frozen in liquid nitrogen (N_2) on day 7, 14 and 21, and stored at -80°C prior usage. 3D scaffolds were then grinded with a pre-cooled mortar with addition of liquid N_2. 1 ml TRI (Molecular Research Center, Cincinnati, MA, US) reagent was added and phase-separation was done according to the instructions of the manufacturer. The supernatant containing the total RNA was then transferred onto Genelute™ silicone purification columns (Sigma-Aldrich) and purified according to the kit's instructions. Total RNA was quality-checked for integrity and purity by Experion™ automated electrophoresis System (Bio-Rad, Reinach, Switzerland). Total RNA (100-200 ng) was used for reverse-transcription (RT) and subsequent

PCR (real-time-qPCR) using gene-specific primers.[6] Oligonucleotide primers (all from Microsynth, Balgach, CH) were designed with Primer Beacon® Designer™ Software (Premier Biosoft Inc., Palo Alto, CA, USA) using nucleotide sequences taken from the GenBank database. RT was performed with bimake's All-in-One cDNA Synthesis SuperMix (cat # B24408 bimake.com, Houston, TX, distributed by Lubioscience GmbH, Switzerland). All primers have been previously tested for efficiency and specificity and all amplicons were checked for specificity by melting curve analysis after the run (Table S-1). Real-time PCR was run using SYBR Green® Supermix (Bio-Rad) and read with the CFX96 Touch™ Real-Time qPCR detection system (Bio-Rad). Genes were amplified by a two-step cycling protocol consisting of a melting step at 95° for 15 s and an annealing step at 61°C for 30 s with 45 repeats. Relative gene expression was then quantified by the $2^{\Delta\Delta Cq}$ method and normalized to ribosomal 18S and GAPDH using the HG-DMEM group as negative control[9] as implemented in the CFX Manager™ Software v3.1 (Bio-Rad).

Statistical analysis

PRISM software (version 6.0e, GraphPad, La Jolla, US) was used to analyze the data. Two-way analysis of variance (ANOVA) with Tukey correction for multiple comparisons was used to compare differences between groups. Values are given means ± SD. A p-value < 0.05 was considered statistically relevant. The p-value in the following graphs is indicated by stars (* $p <$ 0.05, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Characterization of PRP

The result of the cell composition analysis is depicted in Fig. 2. In both PRP protocols platelet count was similar. In L-PRP (813.8 ± 233.7 x 10^9/l) as well as in P-PRP (844.1 ± 126.1 x 10^9/l) thrombocytes were increased four-fold compared to whole blood (198.5 ± 42.38 x 10^9/l). Leucocyte count was, as expected, higher in L-PRP (15.91 ± 8.32 x 10^9/l) than in P-PRP (0.43 ± 0.38 x 10^9/l), and whole blood (4.68 ± 1.81 x 10^9/l). Erythrocytes were highest in whole blood (4.12 ± 0.2 x 10^9/l) compared to L-PRP (1.27 ± 1.31 x 10^{12}/l) and P-PRP (0.08 ± 0.03 x 10^{12}/l). A high variance between the different blood donors was noted (Fig. 2).

Effects on cell viability and ECM production

All experimental groups including the positive control (all groups $p < 0.001$) stimulated a three to four-fold increase in cellular activity on day 21 compared to day 7. Additionally, all groups compared to the negative control had significantly increased metabolic cell activity at day 21 (all groups $p < 0.001$) (Fig. 3A). Similar results could be seen in DNA content with significant augmentation in each group from day 7 to day 21 (all groups $p < 0.001$) except in the negative control (Fig. 3B). Neither in metabolic cell activity nor in the DNA content did the experimental groups differ. A decrease was noted regarding hydroxyproline content standardized by DNA mass from day 7 relative to day 14 ($p = 0.01$) and to day 21 ($p = 0.03$) in the negative control. Also the 2.5% PRP groups showed a decline from day 14 relative to day 21 (2.5% L-PRP ($p = 0.004$), 2.5% P-PRP ($p = 0.01$)). No significant changes were seen in the 20% L-PRP group and the positive control (Fig. 3C). Concerning GAG content per DNA a similar phenomenon could be seen with a decrease from day 7 to day 21 in the 2.5% PRP groups (2.5% L-PRP ($p = 0.01$), 2.5% P-PRP ($p = 0.001$)) with no change in the other groups (Fig. 3D).

Relative gene expression of LC-specific phenotype by RT-qPCR

On day 21, a significant rise in the expression of matrix metalloproteinase (*MMP) 3* in the 2.5% L-PRP group compared to all other groups was observed (i.e., 2.5% P-PRP ($p = 0.01$), 20% L-PRP ($p = 0.002$) and the positive control ($p = 0.003$), respectively, Fig. 4H). Furthermore, MMP13 differed significantly in the 20% L-PRP group compared to the 2.5% PRP groups (i.e., 2.5% L-PRP ($p = 0.03$), 2.5% P-PRP ($p = 0.03$), Fig. 4K). Even though a trend to augmented RNA expression of collagen (*COL) 1A2*, scleraxin (*SCX) A*, aggrecan (*ACAN), tenomodulin (TNMD)* across all groups was seen in 20% L-PRP, it was not significant due to the high inter-donor-variability (Fig. 4A, G, E, K).

DISCUSSION

Due to the poor healing capacity of the injured ACL, the knee is predetermined for early premature osteoarthritis independent of a conservative treatment or replacement with a tendon graft, which is todays gold standart.[10] As an alternative therapy to preserve the ACL, primary sutures supplemented by 3D-PRP scaffolds have been proposed to accelerate healing.[3, 11, 12] Only few studies are published, which examine the influence of PRP on human LCs *in vitro* although understanding is fundamental to optimize its use.

Comparisons among studies using PRP are inconclusive and show variable results depending on platelet concentration as well as the inclusion of leucocytes. Interestingly, supra-physiological platelet concentrations provide no additional stimulating effect and are even counterproductive with induction of cell death and abnormal cell arrangement[4,13] although platelet concentration is linear to growth factor release.[5,13] On the contrary, *in vitro* studies show that the lowest tested

PRP concentration, which was 1 x PRP and 0.5 x 10^6 platelets per µl had the highest proliferation rate. In our sample both protocols yielded a 4 x PRP concentration similar to studies using the same protocols.[5, 14] After dilution to 0.1 x and 0.8 x metabolic cellular activity and DNA content increased in all groups except the negative control but in the 0.1 x group ECM decreased over time. Since human muscle, bone and tendon cells, although all part of the musculoskeletal system, react very differently to PRP[15] it is assumed that each cell type has a different optimal concentration and in the case of LCs lower doses of platelets are effective.

It is still very controversial if the addition of leucocytes to PRP is beneficial for regeneration. On the one hand, they are secreting several growth factors such as PDGF-DD and VEGF[18] but on the other hand they release inflammatory cytokines, such as IL6 and TNF-α, that cause catabolic effects on cells and damage tissue.[19] In the L-PRP group leucocytes were three-fold increased compared to whole blood and forty times increased compared to P-PRP. Interestingly, no change in DNA content, cell metabolic activity and ECM production was detected between the L-PRP and P-PRP group.

After 21 days of culture mRNA expression of selected LC markers were examined and normalized to ribosomal *18S* and *GAPDH* using the HG-DMEM group as negative control. L-PRP promoted a higher expression of *MMPs*, which are activated trough the NF-kB p65 pathway induced by *IL6*.[19] *MMPs* play an essential role in remodeling of soft tissue although when over-expressed a catabolic environment is created.[20] In line with our results McCarrel *et al.*[21] showed, that 2.5% L-PRP concentration stimulated *MMP3*, whereas 20% L-PRP promoted *MMP13* expression. Both genes decode for collagenases: *MMP3* for type III collagenase and

MMP13 for type I collagenase.[19] It seems that *MMP* expression is dose depended on leucocyte concentration with no linear correlation. It will be interesting to see if *in vivo* elevated *MMP* concentrations found in acutely injured tissue will be exponentially multiplied by the addition of leucocytes.[20]

A trend in augmented RNA expression in 20% L-PRP of the anabolic genes *COL1A2*, *SCXA*, *ACAN, TNMD,* which decode for collagen Type I, scleraxis A, aggrecan and tenomodulin, was observed. These molecules are fundamental in ligament healing and restoring ECM during the remodeling phase. Collagen type I and type III are the main molecules of the ACL structure responsible for the ligament's tensile strength.[16] An explanation for the trend seen could be the over-expression of *MMP13*, which breaks down among other collagens mainly collagen type I. Consequently COL1A2 as well as COL1A1 are upregulated, which is regulated by scleraxis, to balance collagen matrix production.[17] Tenomodulin,[18] which is a transmembrane glycoprotein, as well as aggrecan,[19] a large proteoglycan stabilizing the collagen network, are induced also through over-expression of SCXA. Murray *et al.*[20] demonstrated also a significant upregulation of pro-collagen gene expression type I and III in cultures with monocytes and PRP compared to PRP alone after 14 days in LCs as a sign of increased turnover.[20]

Several limitations of the current study exist. Clearly, the healing process of the ACL is much more complex than the *in vitro* cell culture environment and the replication of this work should be repeated in *in vivo* animal studies. Furthermore, a study with even a larger sample size is needed considering the inter-donor variability to strengthen the power of this study and confirm the trend in increase of anabolic genes. However, considering the GMP-compliant material

needed, i.e. collagen patches and commercial kits to produce PRP and to evaluate its cyto-compatibility, this might be limited due to budget constraints.

This study demonstrated that static 3D-LC-cultures treated with a high dilution of PRP still stimulate nearly the same metabolic activity as eight times the concentration. Furthermore, we may conclude that the addition of leucocytes did not elevate cell proliferation but selective expression of catabolic genes.

AUTHORS' CONTRIBUTION

K.AM., C.R., M.RD. and F.DA. performed the laboratory work. K.S. and A.SS. helped with the statistical evaluation and editing of the paper and provided funding. G.B. advised and supported the whole project, supervised the research work and provided funding. The original manuscript is not under consideration elsewhere. All authors approved the manuscript and this submission. We have no conflicts of interest to disclose.

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REFERENCES

1. Kiapour AM, Murray MM. 2014. Basic science of anterior cruciate ligament injury and repair. Bone & Joint Research 3:20-31.
2. Mazzocca AD, McCarthy MBR, Chowaniec DM, et al. 2012. Platelet-Rich Plasma Differs According to Preparation Method and Human Variability. Journal of Bone and Joint Surgery-American Volume 94A:308-316.
3. Murray MM, Flutie BM, Kalish LA, et al. 2016. The Bridge-Enhanced Anterior Cruciate Ligament Repair (BEAR) Procedure An Early Feasibility Cohort Study. Orthopaedic Journal of Sports Medicine 4.
4. Giusti I, D'Ascenzo S, Manco A, et al. 2014. Platelet Concentration in Platelet-Rich Plasma Affects Tenocyte Behavior *In Vitro*. Biomed Research International.
5. Yoshida R, Cheng M, Murray MM. 2014. Increasing Platelet Concentration in Platelet-Rich Plasma Inhibits Anterior Cruciate Ligament Cell Function in Three-Dimensional Culture. Journal of Orthopaedic Research 32:291-295.
6. Gantenbein B, Gadhari N, Chan SC, et al. 2015. Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair. World journal of stem cells 7:521-534.
7. Xiao J, Zhang Y, Wang J, et al. 2010. Monitoring of Cell Viability and Proliferation in Hydrogel-Encapsulated System by Resazurin Assay. Applied Biochemistry and Biotechnology 162:1996-2007.
8. Reno C, Marchuk L, Sciore P, et al. 1997. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 22:1082-&.
9. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402-408.
10. Lohmander LS, Englund PM, Dahl LL, et al. 2007. The long-term consequence of anterior cruciate ligament and meniscus injuries - Osteoarthritis. American Journal of Sports Medicine 35:1756-1769.
11. Fleming BC, Spindler KP, Palmer MP, et al. 2009. Collagen-Platelet Composites Improve the Biomechanical Properties of Healing Anterior Cruciate Ligament Grafts in a Porcine Model. American Journal of Sports Medicine 37:1554-1563.
12. Murray MM, Spindler KP, Devin C, et al. 2006. Use of a collagen-platelet rich plasma scaffold to stimulate healing of a central defect in the canine ACL. Journal of Orthopaedic Research 24:820-830.
13. Eppley BL, Woodell JE, Higgins J. 2004. Platelet quantification and growth factor analysis from platelet-rich plasma: Implications for wound healing. Plastic and Reconstructive Surgery 114:1502-1508.
14. Pifer MA, Maerz T, Baker KC, et al. 2014. Matrix metalloproteinase content and activity in low-platelet, low-leukocyte and high-platelet, high-leukocyte platelet rich plasma (PRP) and the biologic response to PRP by human ligament fibroblasts. Am J Sports Med 42:1211-1218.
15. Mazzocca AD, McCarthy MBR, Chowaniec DM, et al. 2012. The Positive Effects of Different Platelet-Rich Plasma Methods on Human Muscle, Bone, and Tendon Cells. American Journal of Sports Medicine 40:1742-1749.
16. Woo SLY, Abramowitch SD, Kilger R, et al. 2006. Biomechanics of knee ligaments: injury, healing, and repair. Journal of Biomechanics 39:1-20.

17. Lejard V, Brideau G, Blais F, et al. 2007. Scleraxis and NFATc regulate the expression of the pro-alpha 1(I) collagen gene in tendon fibroblasts. Journal of Biological Chemistry 282:17665-17675.
18. Shukunami C, Takimoto A, Oro M, et al. 2006. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Developmental Biology 298:234-247.
19. Liu Y, Watanabe H, Nifuji A, et al. 1997. Overexpression of a single helix-loop-helix-type transcription factor, scleraxis, enhances aggrecan gene expression in osteoblastic osteosarcoma ROS17/2.8 cells. Journal of Biological Chemistry 272:29880-29885.
20. Yoshida R, Murray MM. 2013. Peripheral blood mononuclear cells enhance the anabolic effects of platelet-rich plasma on anterior cruciate ligament fibroblasts. Journal of Orthopaedic Research 31:29-34.

1

FIGURE LEGENDS

Figure 1. Design of 3D culture. Human ACL-derived LC were seeded within collagen scaffolds. PRP was placed in a culture insert (high pore density PET-membrane with 0.4 µm pore ∅) which was not permeable for blood cells.

Figure 2. PRP production from fresh whole blood samples comparing two different protocols, L-PRP (leucocyte-rich) and P-PRP (pure) 2h after collection (A) Erythrocytes (B) Leucocytes (C) Thrombocytes. N=9. Two-way ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3. Time-dependent effect of L-PRP and P-PRP on LCs (A) mitochondrial activity (B) proliferation (C) collagen and (D) GAG production from day 7 to day 21. N=5. ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Effects of P-PRP and L-PRP on the relative gene expression of cell phenotype of low-passage primary ACL-derived LCs after 21 days of 3D culture on FDA-approved collagen patches. Values are relative to serum-free control of 3D culture on the same day. N=5. ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 1. List of Primers designed for real time PCR.

mRNA (Gene ID)	Primer pairs
18S (100008588)	F: CGA TGC GGC GGC GTT ATT C, R: GTG GCA GTG ATG GAA
GAPDH (2597)	F: ATC TTC CAG GAG CGA GAT, R: GGA GGC ATT GCT GAT GAT
ACAN (176)	F: CAT CAC TGC AGC TGT CAC, R: AGC AGC ACT ACC TCC TGG A
COL1A2 (1278)	F: TCA CCT ACA GCA CGC TTG, R: GGT CTG TTT CCA GGG TTG
COL2A1 (1280)	F: AGC AAG AGC AAG GAG AAG, R: GGG AGC CAG ATT GTC ATC
COL3A1 (1281)	F: ATA TCA AAC ACG CAA GGC, R: GAT TAA AGC AAG AGG AAC AC
TNC (3371)	F: TCT CTG CAC ATA GTG AAA AAC AAT ACC, R: TCA AGG CAG TGG TGT CTG TGA
SCXA (642658)	F: CAC CAA CAG CGT GAA CA, R: GCA GCG TCT CAA TCT TGG A
TNMD (64102)	F: ACA AGC AAG TGA GGA AGA A, R: GAC GGC AGT AAA TAC AAC AAT
MMP3 (4314)	F: GAC AAA GGA TAC AAC AGG GAC, R: TGA GTG AGT GAT AGA GTG GG
MMP13 (4322)	F: GTT CAA GGA ATC CAG TCT CTC TAT GG, R: TGG GTC ACA CTT CTC TGG TGT TT
MKX (283078)	F: ACC AAG ACC GAG AAG ATA CT, R: GTC ATD ACT GCT TAC GCT AA

Abbreviations: mRNA, messenger RNA; F, Forward primer; R, Reverse primer; 18S, 18S ribosomal RNA; GAPDH, Glyceraldehyde-3-phosphatedehydrogenase, ACAN, Aggrecan, COL1A2, Collagen Type I alpha 2 chain; COL2A1, Collagen Type II alpha 1 chain; COL3A1, Collagen Type III alpha 1 chain; TNC, Tenascin C; SCXA, Scleraxis bHLH transcription factor, TNMD, Tenomodulin, MMP, Matrix metallopeptidase; MKX, Mohawk homeobox

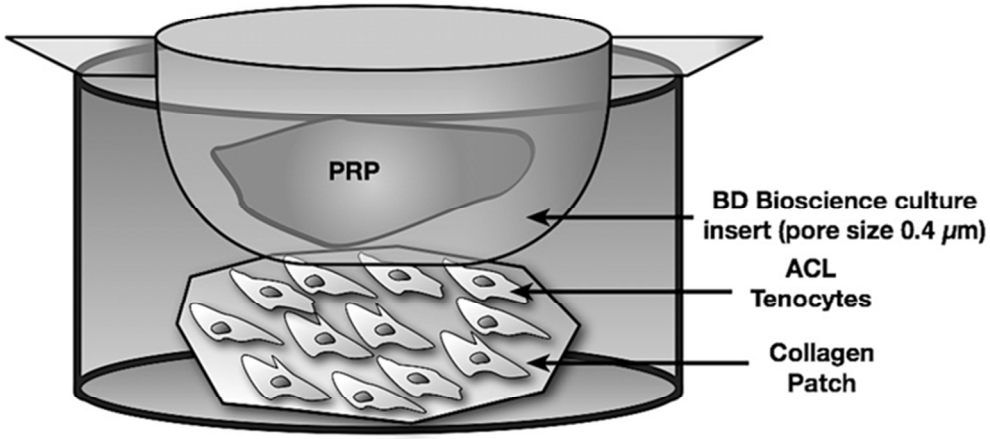

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Figure 1
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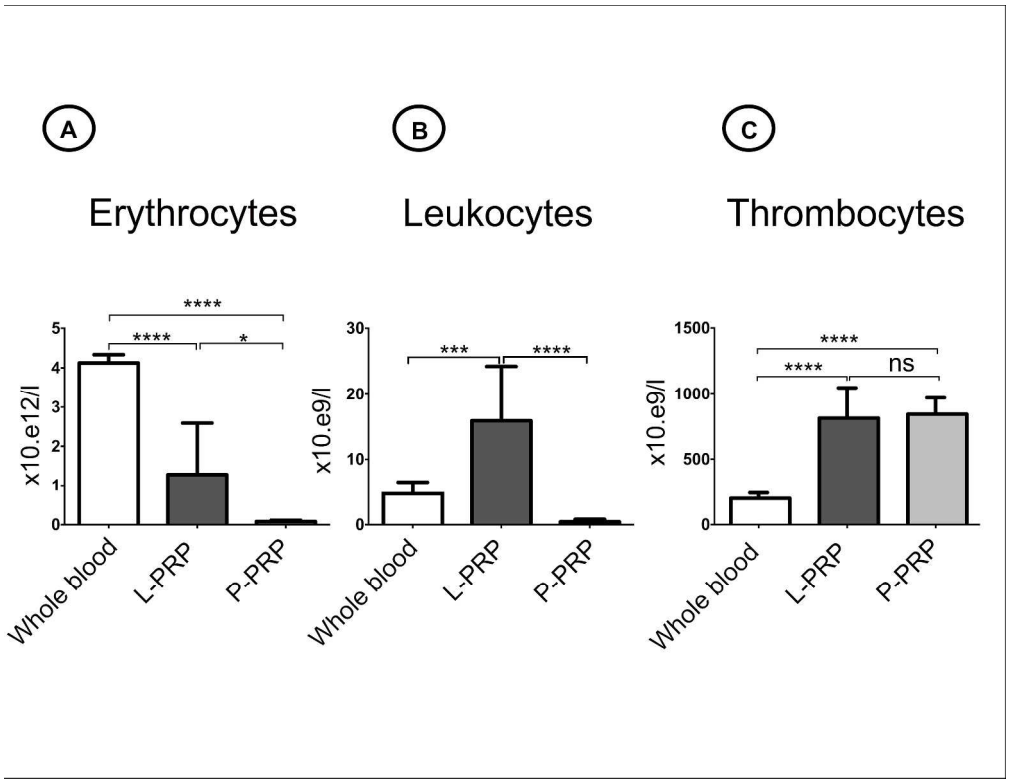

Figure 2. PRP production from fresh whole blood samples comparing two different protocols, L-PRP (leucocyte-rich) and P-PRP (pure) 2h after collection (A) Erythrocytes (B) Leucocytes (C) Thrombocytes. N=9. Two-way ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
Figure 2
337x261mm (300 x 300 DPI)

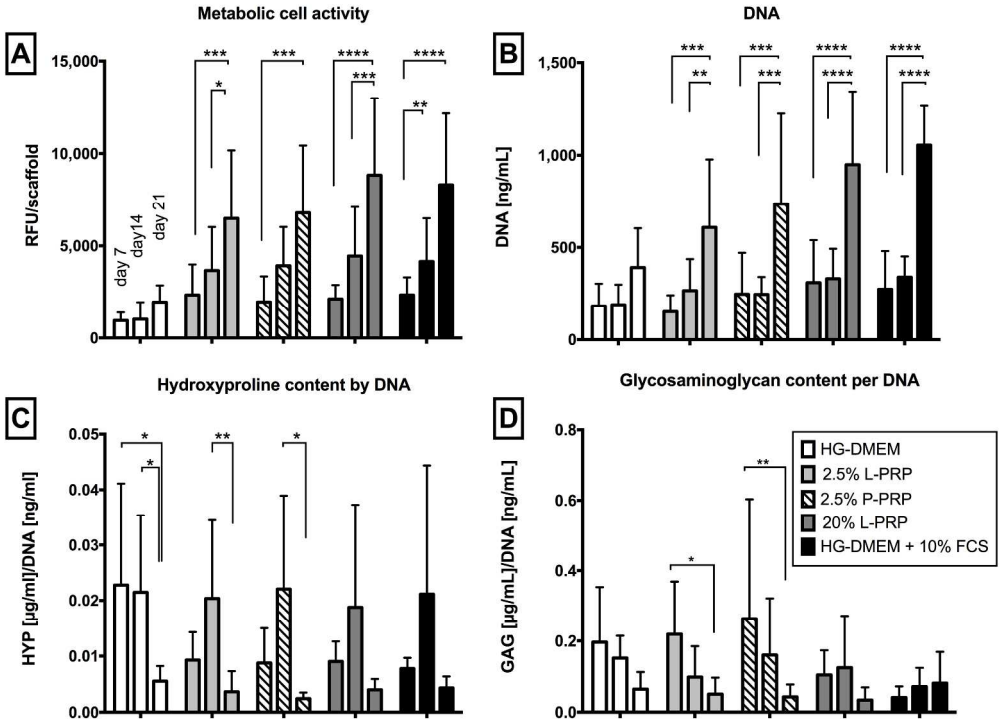

Figure 3. Time-dependent effect of L-PRP and P-PRP on LCs (A) mitochondrial activity (B) proliferation (C) collagen and (D) GAG production from day 7 to day 21. N=5. ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3
255x186mm (300 x 300 DPI)

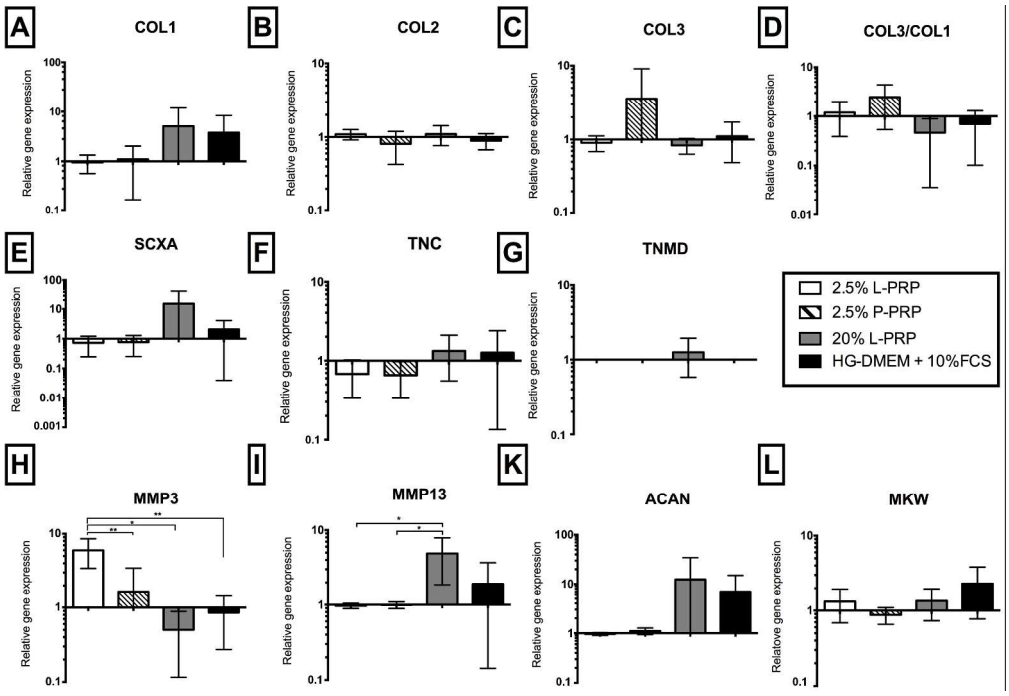

Figure 4. Effects of P-PRP and L-PRP on the relative gene expression of cell phenotype of low-passage primary ACL-derived LCs after 21 days of 3D culture on FDA-approved collagen patches. Values are relative to serum-free control of 3D culture on the same day. N=5. ANOVA was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4
261x179mm (300 x 300 DPI)